Identification of *Pseudomonas syringae* pv. *phaseolicola* by a DNA Hybridization Probe

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ABSTRACT

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A ^{32}P-labeled DNA probe carrying a gene(s) involved in phaseolotoxin production by *Pseudomonas syringae* pv. *phaseolicola* was used to detect and identify *P. s. phaseolicola* in pure or mixed cultures, seed-soak liquids, and diseased specimens collected in the field. The probe hybridized with all 34 strains of *P. s. phaseolicola* tested. All interspecific (pathovar) or intergeneric hybridizations were negative. Hybridization tests were highly reliable for pathogen detection and identification when individual colonies of *P.s. phaseolicola* could be picked individually from seed-soak liquid assay plates or when maceration fluids from disease lesions were assayed. Probings of maceration fluids from disease lesions also were highly reliable. In contrast, soak liquids from seeds contaminated with *P. s. phaseolicola* or washings of colonies from agar plates of such liquids gave variable results.

Additional keywords: diagnosis, halo blight, seed assay.

Pseudomonas syringae pv. *phaseolicola* (Burk.) Young, Dye & Wilkie, the causal agent of halo blight of beans, is a serious seedborne pathogen of beans worldwide (25). The only practical control for this disease is the use of disease-free seed (3,22-25). In an attempt to produce disease-free seed, the industry gradually has moved most seed production to the desert region of south central Idaho. Currently more than 80% of all bean seed produced in the United States is grown in Idaho. Many states and countries have strict quarantines against *P. s. phaseolicola*. In Idaho, seeds entering the state must be assayed and found to be free of *P. s. phaseolicola* and grown in isolation for one season (25). Furthermore, all seed crops are field inspected, and if halo blight is found, the plants must be destroyed. The current laboratory assay used by the Idaho Department of Agriculture involves serology (agar diffusion) (3) and pathogenicity tests. In addition, several commercial seed companies in Idaho assay cull seed samples by a seed-soak/plant inoculation test (24). In England (23), seeds are assayed by plating seed-soak solutions onto medium B of King et al (KB) (8), and in the Netherlands and France a direct immunofluorescent assay is done on the seed soak (24). A modified seed-soak assay in combination with a new semi-selective agar medium (MSP agar) have greatly improved detection of *P. s. phaseolicola* in bean seeds (12). However, even with MSP agar, differentiation of colonies of *P. s. phaseolicola* from other fluorescent pseudomonads can be difficult in some seed lots. Biochemical and pathogenicity tests (18) consequently are needed to confirm identification.

Recently, DNA probes have been developed for the identification of human pathogenic bacteria (17), viroids (1,13), and rhizobia (2). In this report, we describe the use of a cloned DNA fragment from *P. s. phaseolicola* carrying a gene(s) involved in phaseolotoxin production (15) as a specific probe for the detection and identification of the pathogen from seeds and other plant parts. An abstract on the technique has been published (19).

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MATERIALS AND METHODS

Description of the hybridization probe. The DNA fragment used in this study as a hybridization probe was an 8.3-kilobase (kb) DNA fragment cloned from a *tox*::Tn*5* Tox$^-$ mutant, NPS4336, of *P. s. phaseolicola* NPS3121 (15). Cosmid cloning, complementation analysis, Southern blot hybridization (15), and gene replacement experiments (R. C. Peet and N. J. Panopoulos, *unpublished data*) demonstrated that the Tn*5* insertion was located in a 2.6-kb *Eco*R1 fragment and was the cause of the mutation in NPS4336. The 8.3-kb fragment (2.6 kb plus Tn*5*) was initially cloned as part of an approximately 14.3-kb insert in pUC8 to form pRCP1. The 8.3-kb *Eco*R1 fragment from this plasmid was subcloned into pBR322 to form plasmid pRCP2, which was used as the hybridization probe in this study.

Molecular techniques. Total genomic DNA was isolated by the method of Marmur (11). Plasmid pRCP2 DNA was isolated from *Escherichia coli* HB101 by the method of Kahn et al (7). Nick-translation, restriction enzyme digestions, agarose gel electrophoresis, and Southern blot hybridization were done by standard procedures (10). For Southern blot analysis, *Eco*R1-digested genomic DNA fragments from 26 strains of *P. s. phaseolicola*, strain C-271 of *P. s.* pv. *syringae* van Hall, and strain HB101 of *E. coli* were electrophoretically transferred from the agarose gel to a Zeta-Probe nylon membrane (Bio-Rad Laboratories, Richmond, CA) with a Hoefer Transphor Electrophoresis Cell (Model TE 42, Hoefer Instruments Corp., San Francisco, CA). DNA extracts were analyzed by the dot blot hybridization method essentially as described by Kafatos et al (6). For colony hybridization, a variation of the standard method (10) was used to minimize costs: Individual bacterial colonies were grown overnight on KB agar and then transferred with sterile toothpicks onto nitrocellulose membranes (Bio-Rad Laboratories). The cells were lysed by placing the membranes on Whatman 3 MM filter paper impregnated with 10% sodium dodecyl sulfate (SDS) for 5 min. The released DNA was denatured and neutralized by placing membranes in 0.5 M NaOH-1.5 M NaCl for 5 min, followed by 5 min in 1 M Tris-HCl (pH 8.0)-

1.5 M NaCl. The membranes then were baked for 2 hr in a vacuum oven at 80 C. Hybridizations were performed in a solution of 6× SSPE, 0.01 M ethylenediaminetetraacetic acid, 5× Denhardt solution, 0.5% SDS, 100 μg of denatured salmon sperm DNA per milliliter, and 1–2 × 10^6 cpm of ^{32}P-labeled probe at 68 C for 16 hr with gentle rocking (10). Probed filters were washed at room temperature in 2× SSPE-0.5% SDS for 5 min, in 2× SSPE for 5 min at room temperature, and in 0.1× SSPE-0.5% SDS for 2.5 hr at 61 C. Filters were dried, and autoradiography was carried out at −80 C with Kodak XAR-5 film. These conditions were used because in previous work they prevented the development of false positives and were not optimized further. *E. coli* HB101(pRCP2) and *E. coli* HB101 were used as positive and negative controls, respectively.

Specificity of hybridization probe. The following bacterial strains (number refers to number of strains) were tested for homology to the probe fragment by the modified colony hybridization method above: *P. s. phaseolicola*, 34; *P. s. syringae* from beans, 71; *P. s. syringae* from citrus, 2; *P. s. tabaci*, 7; *P. s. pisi*, 9; *P. s. viridiflava*, 5; *P. s. tomato*, 5; *P. s. coronafaciens*, 2; *P. s. glycinea*, 5; *P. s. lachrymans*, 1; *P. s. angulata*, 2; *P. s. maculicola*, 2; *P. s. marginalis*, 4; *P. s. andropogonis*, 1; *P. s. corrugata*, 1; *P. s. atrofaciens*, 2; *P. s. cichorii*, 1; *P. solanacearum*, 1; *Xanthomonas campestris* pv. *phaseoli*, 1; *X. c. vesicatoria*, 1; *Rhizobium japonicum*, 1; unknown oxidase positive pseudomonads isolated from beans, 28; and 21 other bacteria. Bacteria were from the collections of N. W. Schaad, N. Panopoulos, and M. N. Schroth.

Sensitivity of bulked cell suspension and DNA dot blot assays. To simplify the use of the method at reduced cost, hybridizations were carried out with "bulked" cell suspensions washed from agar plates as well as with DNA extracts for such suspensions as follows. Dilutions of a liquid culture of strain C-199 of *P. s. phaseolicola* were spread on KB or MSP agar media to give 1–2, 5–10, and 30–40 colonies per Petri plate. The plates were incubated for 48 or 96 hr at 23–25 C. Each plate was washed four times with 5 ml of sterile saline using an L-shaped glass rod and turntable. The pooled washings (20 ml) were considered as 1× samples. Aliquots of 1.5 ml were removed from these 1× samples for DNA extraction and 18-ml aliquots were centrifuged at 10,100 *g* for 10 min. The pellets were suspended in 1.8 ml of saline to give more concentrated (10×) samples. Aliquots of 1.5 ml from 10× samples also were used for DNA extraction. Ten-microliter aliquots of 1× and 10× samples and their corresponding DNA extractions were spotted onto nitrocellulose membranes for bulked cell suspension and dot hybridization assays, respectively.

Recovery and detection of *P. s. phaseolicola* added to healthy bean seeds. Three thousand bean seeds (*Phaseolus vulgaris* L. 'Viva'), previously shown to be free of *P. s. syringae* and *P. s. phaseolicola* by seed assays (12), were soaked at 4 C in 3,000 ml of sterile saline for 24 hr. A suspension (1.0 OD at 600 nm) of strain C-199 of *P. s. phaseolicola* grown for 24 hr in liquid medium 523 (5) was serially diluted (10^3, 10^4, 10^7), and 1-ml aliquots from each dilution were added to 99 ml of the above seed washings. After mixing thoroughly, 10-fold serial dilutions were plated onto KB and MSP agar plates. In parallel, 1-ml aliquots of the same dilutions of C-199 cells were diluted in 99 ml of saline and plated as above to determine actual numbers of viable cells present. Dilutions of the seed washings to which no C-199 cells had been added were similarly plated. Plates were incubated at 23–25 C for 96 hr. Individual bacterial colonies or bulked cell washings were transferred to nitrocellulose membranes and probed as described earlier.

Detection of *P. s. phaseolicola* in naturally contaminated bean seeds. A 50-g sample of bean seed, cultivar Red Kidney, naturally contaminated with *P. s. phaseolicola* (received from A. W. Saettler, Agricultural Research Service, U.S. Department of Agriculture, Michigan State University, East Lansing) was assayed by the seed-soak/MSP agar plating method, as described (12). A 250-g sample of cull seed determined to be free of *P. s. phaseolicola* by previous seed-soak assays (12) also was assayed in the same manner. The ratio of colonies of *P. s. phaseolicola* to colonies of saprophytes growing on the MSP agar plates was varied by mixing washings from the Red Kidney and cull seeds in different ratios. After 96 hr at 23–26 C, plates with different numbers of *P. s. phaseolicola* and other bacterial colonies were selected. The plates were washed with 20 ml of saline. Of the resulting suspension, 10-μl aliquots were spotted onto a nitrocellulose membrane for bulked cell hybridization and 1.5 ml was centrifuged in a microfuge. The pellet was suspended in 150 μl of saline, and 10-μl aliquots were spotted onto a nitrocellulose membrane for hybridization as described above.

Detection of *P. s. phaseolicola* in plant tissue. Lesion tissue from dried mature bean pods with symptoms of halo blight and/or brown spot collected from fields in 1984 in southern Idaho was removed and macerated with a scalpel in a drop of sterile saline in a plastic Petri plate. For negative controls, healthy leaves and leaves with injury-type lesions collected the same day from a bean field at Moscow were soaked in saline for 5 min as above. Ten-microliter aliquots of each sample were spotted onto nitrocellulose membranes for hybridization. To verify the presence or absence of viable cells of *P. s. phaseolicola* and/or *P. s. syringae* in the pod samples, the maceration liquids were streaked with a loop onto MSP and KBC, media semiselective for *P. s. phaseolicola* and *P. s. syringae*, respectively (12).

Phaseolotoxin assay and plant inoculations. Phaseolotoxin production was determined by the microbiological assay described previously (20). Bacteria were grown at both 18 and 28 C for 2 days in minimal-glucose medium (15), and the plates were overlaid with a log-phase culture of *E. coli* strain PB1576 (prototrophic) (from D. Lane, Dept. of Cell Biology, University of Auckland, New Zealand). *P. s. phaseolicola* NPS3121, which produces phaseolotoxin, was used as the toxin-positive control. Bean plants (cultivar Viva) at the two- to three-leaf stage were inoculated by the pinprick method (9). Strain C-173 also was tested for pathogenicity on soybean as above.

RESULTS

Specificity of the DNA probe. Previous studies (15) had indicated that the 2.6-kb DNA insert of plasmid pRCP2 hybridized only to DNA of *P. s. phaseolicola*. However, the number of bacterial strains used in those studies was limited. Therefore, in the present study we sought to establish the specificity of the probe by examining a larger number of strains. Of the 207 strains tested, the 34 strains of *P. s. phaseolicola* all gave a positive hybridization signal when tested according to the modified colony hybridization procedure.

The other 173 strains, representing closely and distantly related bacteria, were all negative in our tests. An apparent exception occurred with one strain (C-173) which had been maintained in our collection as *P. s. glycinea* and which gave a positive signal in hybridization tests. The pathovar identity of this strain was examined by inoculation tests as well as by the microbiological assay for phaseolotoxin (20). The strain infected bean causing halo symptoms but did not infect soybeans. Strain C-173 also caused citrulline-reversible inhibition of *E. coli*, which is indicative of phaseolotoxin production. As with phaseolotoxin-producing strains of *P. s. phaseolicola*, inhibition of *E. coli* occurred when strain C-173 was grown at 18 C before the bioassay and not when grown at 28 C. Based on these results, the classification of this strain as *P. s. glycinea* is considered incorrect.

Southern blotting of *Eco*R1-digested DNA from 26 of these strains further showed that the homology to pRCP2 was associated with a 2.6-kb fragment, as in the strain from which the toxin gene fragment was originally derived (15). No homology was observed with DNA from *P. s. syringae* and *E. coli* HB101 (data not shown).

Sensitivity of the bulked cell suspension and DNA dot blot hybridization assays. Single colonies of *P. s. phaseolicola* were detected consistently by hybridization of the probe to bulked cell suspensions from plate washings from MSP agar medium provided that the bacteria had been allowed to grow on these

plates for 96 hr but not when the bacteria were allowed to grow only for 48 hr (Table 1). A positive hybridization signal obtained with DNA of *P. s. phaseolicola* was easy to distinguish from a negative signal obtained with DNA of *P. s. syringae* (compare Fig. 1, rows 1 and 2, column D, with rows 3 and 4, column D, respectively). Compared with bulked cell hybridization, dot blot hybridization with DNA extracted from plate washings was less reliable, especially when the plates contained few colonies. For example, plates that contained one or two colonies and were incubated for 96 hr were scored positive by the bulked cell suspension method when unconcentrated (1×) suspensions were used (Table 1). However, DNA dot blot assays under these conditions gave negative results (data not shown). We presume that this reflects low or variable yield of DNA during extraction from dilute cell suspensions; more concentrated DNA extracts or larger aliquots were not tested in this study.

TABLE 1. Detection of *Pseudomonas syringae* pv. *phaseolicola* by hybridization with "bulked" cells washed from Petri plates

No. of colonies of *P. s. phaseolicola* per plate	No. of positive samples[a]			
	48-hr-old colonies		96-hr-old colonies	
	1×	10×	1×	10×
1–2	0/6[b]	0/6	6/6	6/6
5–10	1/3	1/3	3/3	3/3
30–40	0/3	3/3	3/3	3/3

[a]The MSP (12) agar plates were incubated at 23–25 C and washed with 20 ml of saline as described in the text. Ten-microliter aliquots of this original sample and a sample concentrated 10× were spotted on a nitrocellulose membrane. Cells were lysed and hybridized with ^{32}P-labeled pRCP2, as described in the Materials and Methods section.
[b]Number of positive samples per total number of samples tested.

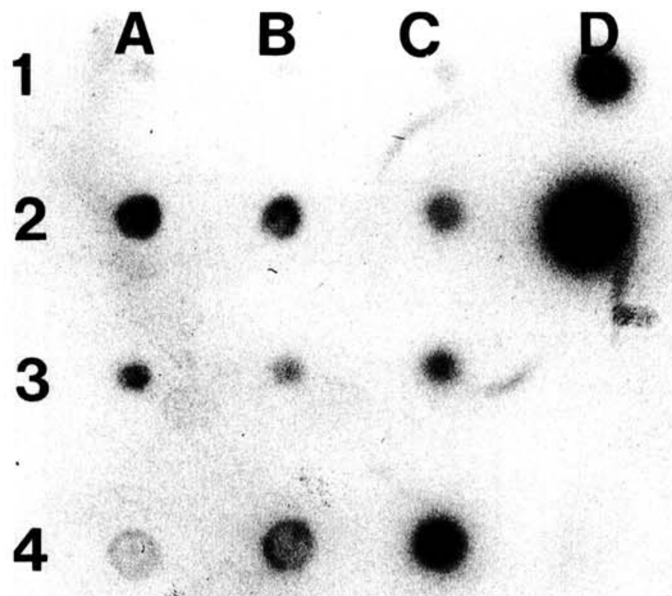

Fig. 1. Autoradiograph of "bulked" cell suspension and DNA dot hybridization of cells of *Pseudomonas syringae* pv. *phaseolicola* and *P. s. syringae* after 96 hr incubation, probed with ^{32}P-labeled pRCP2 DNA: 1) washings from bean seeds naturally contaminated with *P. s. phaseolicola* (rows 1, 2, and 3, columns A, B, and C); 2) dot hybridizations of pure cultures of *P. s. phaseolicola* (row 4, columns A, B, and C); and 3) DNA from *P. s. phaseolicola* (rows 1 and 2, column D) and *P. s. syringae* (rows 3 and 4, column D). Row 1, columns A, B, and C, contains a mean of 3.3 colony-forming units (cfu) (*P. s. phaseolicola*/176 cfu of saprophytes). Row 2, columns A, B, and C, contains 216 cfu (*P. s. phaseolicola*/47 cfu of saprophytes). Row 3, columns A, B, and C, contains 14 cfu (*P. s. phaseolicola*/184 cfu of saprophytes). Row 4, columns A, B, and C, contains, respectively, washings of plates containing 2, 6, and 25 cfu of a pure culture of *P. s. phaseolicola*. Rows 1 and 2, column D, and rows 3 and 4, column D, contain DNA from *P. s. phaseolicola* (positive control) and *P. s. syringae* (negative control), respectively. Rows 1 and 3, column D, and rows 2 and 4, column D, contain approximately 1.0 and 2.0 μg of DNA, respectively.

Detection of *P. s. phaseolicola* added to washings of healthy bean seeds. *P. s. phaseolicola* could not be detected by bulked cell suspensions of 1× washings (Table 2). Only when washings were concentrated 10× and numbers of saprophytes were relatively low (around 100 colonies or less) was the presence of the pathogen detected reproducibly by this method (Table 2). Results were always positive when colonies of *P. s. phaseolicola* were clearly separate and recognizable on the MSP agar plates and were transferred by toothpicks to filters (data not shown). As might be expected, when large numbers of saprophytes are present on MSP agar, fewer colonies of the pathogen develop or are observed on these plates. Therefore, sensitivity of the bulked cell suspension hybridization method in those situations is reduced (Table 2). In only one case was hybridization to bulked cell suspension positive when no distinct colonies of *P. s. phaseolicola* were observed (310–450 saprophytes, Table 2).

Detection of *P. s. phaseolicola* in naturally contaminated bean seeds. The presence of the pathogen in naturally contaminated seed lots could not be detected by probing 1× washings from MSP agar plates (data not shown). However, probing of 10× washings from such plates gave mostly positive results (Table 3 and Fig. 1, rows 1, 2, and 3, columns A, B, and C). Low-level positive results of bulked cell suspensions (row 1, columns A, B, and C) and negative results (rows 3 and 4, column D) were easily distinguished (Fig. 1). All plates containing 17–25 and 13–19 colonies of *P. s. phaseolicola* and 28–66 and 174–204 colonies of saprophytes, respectively, were positive (Table 3). However, when plates contained only 1 or 0–6 colonies of *P. s. phaseolicola* and approximately 50 or more colonies of saprophytes, the results were highly variable (Table 3).

Detection of *P. s. phaseolicola* in plant tissue. All pods with halo-blight-like lesions were positive for *P. s. phaseolicola* both by the MSP agar plate method and by bulked cell suspension hybridization (Table 4). Furthermore, *P. s. phaseolicola* was detected in two of five and four of five pods with brown-spot-like lesions by both methods. All healthy samples and those with mechanical injury or lesions atypical of bacterial blights were negative.

DISCUSSION

A DNA probe containing a fragment carrying a gene(s) involved in phaseolotoxin production by *P. s. phaseolicola* hybridized with DNA isolated from all strains of *P. s. phaseolicola* tested. Only one other bacterium of a total of 173 strains that were tested gave a positive hybridization signal. However, inoculation tests showed that this strain (C-173) infected beans but not soybeans.

TABLE 2. Detection of *Pseudomonas syringae* pv. *phaseolicola (P.s.p.)* cells added to healthy bean seeds

No. of colonies of *P.s.p.* expected per plate[a]	No. of colonies of *P.s.p.* observed per plate	No. of colonies of saprophytes per plate	Detection of *P.s.p.* by "bulked" cell suspension[b]	
			1×	10×
3	1	83–120	0/3[d]	2/3
3	2–5	64–129	0/6	6/6
3	0	310–450	0/3	1/3
23	15–21	91–109	0/3	3/3
23	12–18[c]	143–121	0/3	1/3
23	2–4[c]	308–416	0/3	1/3

[a]Figures based upon addition of *P. s. phaseolicola* to saline and plating onto medium B of King et al (8), three replicates of each.
[b]The MSP (12) plates were washed after 96 hr at 23–25 C, and samples were spotted onto nitrocellulose membrane. Hybridization was with ^{32}P-labeled pRCP2 as described in the Materials and Methods section.
[c]Most colonies of *P. s. phaseolicola* were considerably smaller than normal due to the presence of other bacterial colonies.
[d]Number of positive samples per total number tested.

Furthermore, strain C-173 produced a phaseolotoxin-like inhibitor. Whether the strain was misidentified initially or simply was mislabeled is not known. The results, therefore, show that the probe used in this study is reliable for the identification of *P. s. phaseolicola*. The hybridization signal detected with DNA from C-173 as well as the other 34 strains that tested positive in our experiments is attributed to homologous sequences contained within the 2.6-kb tox fragment. Thus, the homologous fragment detected was of equal length (2.6 kb) to the tox fragment contained in the probe, and no restriction fragment length polymorphisms were detected in any of the 26 geographically diverse strains of *P. s. phaseolicola* tested or in strain C-173. Furthermore, neither pBR322 nor Tn*5*-containing probes hybridize to DNA of *P. s. phaseolicola* (15). The hybridization therefore is due to the tox gene fragment and not the Tn*5*.

The correct identification of *P. s. phaseolicola* requires biochemical and pathogenicity tests (18). The seed-soak/plant inoculation (25) test is an improvement over Idaho's presently used serological method (3) of screening bean seed lots for possible contamination with *P. s. phaseolicola*. Serological methods provide a rapid identification of bacteria; however, cross-reactions with saprophytic bacteria associated with bean often are observed (N. W. Schaad, *unpublished data*). DNA probes have been used for the identification of plant viroids (1,13), rhizobia (2), and bacteria of medical importance (17). Because *P. s. phaseolicola* is the only organism known to produce phaseolotoxin (20) and the probe used appears to be specific for the bacterium, DNA hybridization can be applied for the correct identification of this pathogen.

Plasmid pRCP2 was used successfully as a hybridization probe for the detection of *P. s. phaseolicola* in infected plant tissue and for the rapid identification of colonies of *P. s. phaseolicola* growing on a semiselective medium (MSP). However, detection of the pathogen in contaminated seed depended on several variables. Thus reliable detection by the bulked cell suspension hybridization method required more than 48 hr prior incubation of the MSP agar plates. Most likely, the amount of the tox gene DNA extractable from pooled colonies less than 96 hr old and/or its proportion to total DNA is not enough to be detected with probes labeled by nick-translation. Higher specific activity probes might be obtained in several ways: by using probes that do not contain extraneous sequences, by labeling with random primers, by using in vitro transcribed RNA probes, or by using the DNA polymerase chain reaction. Better detection also might be obtained with rRNA gene probes (D. Roth, *unpublished*). The successful detection of *P. s. phaseolicola* among cells washed from the semiselective medium also depended on the ratio of colonies of *P. s. phaseolicola* relative to saprophytes growing on the MSP plates. An advantage of MSP agar over nonselective KB is that fewer colonies of saprophytes were present and colonies of *P. s. phaseolicola* continue to grow upon further incubation whereas those of most saprophytes do not. Thus, both the amount of DNA of *P. s. phaseolicola* and the proportion of *P. s. phaseolicola* to DNA originating from other bacteria growing on these plates should change favorably with increased incubation time.

TABLE 3. Detection of *Pseudomonas syringae* pv. *phaseolicola* in naturally contaminated bean seeds

Range of colony-forming units observed per plate[a]		Number of samples positive by hybridization with "bulked" cell suspensions[b]
P. s. phaseolicola	Saprophytes	
0–6	30–56	1/3
1	56–89	1/3
1–4	56–95	3/3
1–5	154–198	3/3
17–25	28–66	3/3
13–19	174–204	3/3

[a]Seeds were washed and 0.1 ml of washing was plated onto each of three plates of MSP agar as described (12). Colonies were determined to be *P. s. phaseolicola* by colony morphology and presence of blue fluorescent pigment.

[b]Plates were incubated for 96 hr at 23–25 C. Plate washings were concentrated 10× and probed with ^{32}P-labeled pRCP2 as described in the Materials and Methods section.

TABLE 4. Detection of *Pseudomonas syringae* pv. *phaseolicola* in plant tissue

Sample	Isolation onto semiselective agar medium for: *P. s. syringae*	*P. s. phaseolicola*	"Bulked" cell suspension hybridization
Leaves, healthy	0.2[a]	0/2	0/2
Leaves, lesions (not typical)	0/3	0/3	0/3
Pods, lesions (brown spots)	0/5	2/5	4/5
Pods, lesions (halo blight)	0/3	3/3	3/3

[a]Number of positive samples per total number of samples tested.

The DNA probe used in this study has proven very useful for rapid identification of *P. s. phaseolicola* in our seed assay (12). The ability to quickly differentiate *P. s. phaseolicola* from *P. s. syringae* and saprophytic fluorescent pseudomonads also will aid in studying the ecology of *P. s. phaseolicola*. In addition, the hybridization probe will be very useful for the direct diagnosis of halo blight in the field. Lesions can be removed from field samples, macerated in a droplet of water, and spotted onto a membrane. The sample can be mailed to a laboratory to be tested. Apparently there are large enough numbers of cells of the pathogen and few enough cells of saprophytes present in the halo blight lesions to yield enough DNA to be detected by the probe. Such sampling may be useful for field inspections in seed certification programs. An added advantage of using this or other tox gene probes for identification of colonies of *P. s. phaseolicola* is that nonfluorescent strains of the pathogen are easily identified. Often such strains go unidentified in routine KB medium isolation. Although the probe was not tested against the full range of the organisms that might be on seeds, no bacterium of those tested, other than *P. s. phaseolicola*, gave a positive hybridization signal with plasmid pRCP2. The presence of Tn*5* and vector sequences in pRCP2 could result in positive hybridization with enteric bacteria such as *Erwinia* sp. However, the chance of that happening would be remote because enteric bacteria do not grow on media with boric acid. Even if growth did occur on MSP, enteric bacteria could be easily differentiated from *P. s. phaseolicola*. Still, a toxin gene recloned in a smaller vector without Tn*5* would provide an even more specific probe.

The presence of Tn*5* and vector sequences in pRCP2 make this plasmid inappropriate for routine use in the testing of seed lots of field specimens. After this study was carried out, the wild-type 2.6-kb segment comprising a portion of the pRCP2 insert as well as several additional tox gene segments were cloned individually and can be made available for these purposes.

Available evidence suggests that genes involved in phaseolotoxin production are not plasmid encoded (4,14,16). The genes thus far identified do appear to be clustered in the genome of *P. s. phaseolicola* (14). Ideally, several different cloned fragments spanning the phaseotoxin gene cluster should be used as hybridization probes to insure detection of Tox$^-$ mutants that might carry partial deletions in this region. Naturally occurring Tox$^-$ strains have not been observed in Idaho; however, such strains are known to occur occasionally in other parts of the world (N. J. Panopoulos, *unpublished*).

A major disadvantage of radioisotope-labeled probes, in addition to cost, is the need for approved laboratory facilities and trained personnel. A nonradioactive label would greatly increase the usefulness of the method. We have done preliminary tests with biotin and immunosulfonated DNA (21) labels. Although less sensitive than radioactive labels, such labels should be sensitive enough to be used for colony hybridization of single

colonies (i.e., identification) transferred to membranes by toothpicks.

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